Localization and Proof of Chitin in the Opisthobranch Mollusks *Aplysia californica* Cooper and *Bulla gouldiana* (Pilsbry), with an Enzymochromatographic Method for Chitin Demonstration

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CHITIN HAS been generally considered as almost synonymous with the arthropod exoskeleton. However, as Richards (1951) points out, other substances enter into the structure of the arthropod exoskeleton and, conversely, some arthropods are known which have no detectable chitin. It is also true that chitin is found in other invertebrate phyla and in the plant kingdom as well. Wester (1909) and Kunike (1925) surveyed the animal kingdom for chitin and reported it present in at least some part of the body of members of most of the phyla below the echinoderms. These and the other authors mentioned below found chitin present in four of the five molluscan orders (Scaphopoda was not studied). Besides these works Sollas (1907), Spek (1919), Pantin (1925), and Tóth (1940) have contributed to the study of chitin in the Gastropoda. Chitin has been reported present in only two structures, the radula and the jaws. Tóth (1940) reported that the organic portion of the shell of *Helix pomatia* did not contain chitin. Kunike (1925) found no chitin in the organic shells of the gastropods and lamellibranchs which he tested. Roche (1951) hydrolyzed the organic residues of the gastropod shells of *Turbo, Meleagrina,* and two species of *Pinna,* and reported them to be scleroproteins. Spek tested the radulae and jaws of a number of common prosobranchs and pulmonates and of the opisthobranch *Aplysia* sp., and found chitin present in all cases. The radulae and jaws of the genera which have been tested in these works include *Helix, Arion, Patella, Haliotis, Natica, Conus, Buccinium, Littorina, Tergipes,* and *Aplysia.*

As Richards (1951) points out, it is essential that we establish a criterion for the demonstration of the polysaccharide chitin. He accepted a positive chitosan color test as the most valid qualitative test for the presence of chitin. This test is also the standard used in this paper, although the chromatographic demonstration of N-acetylglucosamine, the basic saccharide of chitin, would also be an adequate demonstration of the presence of chitin.

Besides the two chitinous structures previously studied, the opisthobranch *Aplysia,* commonly known as the sea hare, possesses two other "chitinous" structures, the stomach teeth and a vestigial shell, both of which are composed almost entirely of organic material. The shell-bearing opisthobranch, *Bulla,* possesses stomach teeth and a small amount of organic periostracum on its shell. These structures have been tested by the chitosan color test method. In addition, an enzymochromatographic method for chitin demonstration was adapted and tested and is herewith presented. The shell of the prosobranch *Oncomelania* was also tested for comparison.

MATERIALS AND METHODS

Stomach teeth were excised from growing specimens of *Aplysia californica* Cooper and frozen until ready for testing. Shells of adult specimens were removed and preserved in 70 per cent ethyl alcohol until tested.

Adult specimens of the shell-bearing opisthobranch, *Bulla gouldiana* (Pilsbry), were collected and preserved in neutral formalin (which was later changed to 70 per cent ethyl alcohol). When the test was made the shells were decalcified in 10 per cent HCl, rinsed well, and blotted. The dry shells of *Oncomelania nosophora* (Robson) were similarly decalcified.

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The difference in preparation was a result of convenience. Commerically prepared chitin was used for controls in all cases.

The chitosan color test of Campbell, as adapted by Richards (1951), was used as follows:

The chitin to be tested was placed in a test tube and a saturate solution of KOH was added. The tube was closed with a Bunsen valve (a piece of rubber tubing sealed at one end with a clamp and having a longitudinal slit 0.5–0 cm. long made on its lateral surface). The test tube was then placed in a glycerine bath and heated to 160° C. over a period of 15–20 min. It was held at this temperature for about 15 min. and allowed to cool to room temperature. The persistent material assumed to be chitosan[2] was then rinsed and divided for the following tests:

(1) Lugol's iodine was added, resulting in a brown color when positive. The excess iodine was removed and replaced with 1 per cent sulfuric acid. When chitin was present it changed color to what appeared black. However, upon mashing the specimen under a microscope, the true color—a reddish purple—was apparent (the chitosan color test). Adding 75 per cent sulfuric acid resulted in an immediate change to brown, followed by an eventual disappearance of the color as the material passed slowly into solution. (2) Three per cent acetic acid was added to another portion of the original KOH persistent material. When positive the tested substance passed slowly into solution. Addition of a drop of 1 per cent sulfuric acid then gave a white precipitate.

For the enzymochromatographic determination the chitin was mashed in a mortar or minced and placed in a miniature test tube. To this 1 ml. of snail enzyme preparation was added, after which it was incubated overnight at room temperature. After incubation small samples were applied in spots about 1½ in. apart on a line ½ in. from the base of 8 × 8-in. squares of Whatman No. 1 chromatographic paper. The paper was then formed into a cylinder and fastened with staples so that the base line described the circumference at one end. This end was then placed in a Petri dish containing water–saturated phenol (Mallinckrodt liquid) which was not deep enough to reach the sample. This in turn was placed in a 10-gal. aquarium and covered with a glass plate. When development was completed the paper was dried overnight and then washed in ether to remove all the phenol possible. After further drying the chromatogram was dipped in benzidine-trichloroacetic acid reagent and heated at 100° C. for about 15 min., or until the brown spots indicating reducing substances had thoroughly formed. The spots disappear after a few days so it is necessary to mark the spots and soak the papers in water to have a permanent record.

The snail enzyme reagent was prepared by excising the digestive gland of the common garden snail, *Helix aspersa,* and macerating it in a mortar with sand and acetate buffer at pH 5.2. It was then centrifuged and the liquid was used in the digestion as described.

The benzidine-tricholoracetic acid reagent was made as follows: 0.5 gm. of benzidine was dissolved in 5 ml. of glacial acetic acid and added to 4 gm. of trichloroacetic acid dissolved in 5 ml. of water. To this was added 90 ml. of acetone. If refrigerated it keeps about 2 weeks.

EXPERIMENTAL RESULTS

Both the nuclear area of the shell and the stomach teeth of *Aplysia californica* were resistant to the action of the hot alkali and gave very strong chitosan color tests. The confirmatory tests mentioned were also positive. The organic periostracum of *Bulla* contained small amounts of chitin although the major portion was not persistent. The stomach teeth of *Bulla* were composed principally of chitin. The decalcified organic shell of *Oncomelania,* however, did not withstand the action of the hot alkali. Some white powder released was tested but gave no color. This latter shell material was completely resistant to the snail enzymes.

Chromatographic spots at Rf .34 for all specimens and controls involving the snail enzyme preparation were the result of free sugars in the enzyme extract. The additional spots at an Rf value of approximately .69 appeared only in the aplysiid material tested and the chitin con-

[2] Few organic substances other than chitin can resist the KOH treatment.

trols. These represent the spots resulting from the presence of N-acetylglucosamine, the building block of chitin.

DISCUSSION AND CONCLUSION

The presence of chitin as the principal constituent of the stomach teeth may not be surprising, considering that the hard structures of the anterior digestive tract of all gastropods tested have been shown to consist of chitin. However, the organic portion of the gastropod shell has consistently been reported negative for chitin in other snail groups tested and has been found so in this investigation in the amphibious prosobranch *Oncomelania.* The nature of the shell and the relative proportions of calcium and organic matter are very different in the gastropods possessing calcareous shells as against the largely organic, vestigial shell of most opisthobranchs. At most the amount of organic matter in the calcareous type of shell of the former constitutes a periostracum or matrix. However, in many of the latter the vestigial shell is almost, if not entirely, composed of organic material. In the shell-bearing opisthobranch, *Bulla,* the organic matter is considerably less than in *Aplysia* and can be compared to that of the pulmonates, such as *Helix* and *Vivaparus.* The amount of calcium present in the aplysiid shell varies, some possessing little, if any, apparent calcareous layer, while in others a calcareous film or crust is formed on the inner surface of the organic shell. In *A. californica* rarely more than a translucent film is present. The presence of an occasional thicker calcareous layer in this latter species seems to be associated with a diet high in *Corallina* sp., a calcareous alga. Some species are said to normally deposit a calcareous layer or crust.

The enzymochromatographic method of chitin detection here adapted from existing techniques offers one very distinct advantage. As suggested by Richards (1951), there may be chitins in a broader sense which are not persistent in the hot KOH treatment used in the chitosan color test and therefore are not detected even though they are made up of the requisite N-acetylglucosamine. These would be detected by the enzymochromatographic method described unless they are not attacked by the snail enzyme—which is unlikely.

The presence of chitinase in the digestive gland of the large European snail, *Helix pomatia,* has been exploited in the past for the enzymatic dissolution of chitin (Karrer, 1930). Jeuniaux (1954) found a chitinase present in the digestive organs of *H. aspersa,* the common garden snail.

SUMMARY

The stomach teeth of *Aplysia californica* and *Bulla gouldiana* were tested for chitin by the specific chitosan color test method and were found to be strongly positive. The organic shell of *Aplysia californica* gave a positive chitosan and confirmatory test. The organic periostracum of *Bulla gouldiana* left some KOH–resistant material, giving positive chitosan color and confirmatory tests. The organic portion of the shell of the amphibious prosobranch, *Oncomelania,* was not KOH–resistant and was negative for chitin. The stomach teeth of *Aplysia* were tested by enzyme digestion, followed by chromatographic analysis of the soluble residues, and showed the presence of N-acetylglucosamine comparable to that found in the commercial chitin used as controls.

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